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Applicant:	Teicher et al.	Art Unit:	1654
Serial No.:	10/646,063	Examiner:	Marcela M. Cordero Garcia
Filed:	August 22, 2003	Customer No.:	21559
Title:	CORTICOSTEROID CONJUGATES AND USES THEREOF		

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DECLARATION OF DR. MARTIN H. TEICHER UNDER 37 C.F.R. § 1.132
TRAVERSING GROUNDS OF REJECTION

Under 37 C.F.R. § 1.132 and regarding the rejection of claims 12, 13, 15, 19-23, and 29-30, in view of Zhang et al. (J. Pharm. Sci. 90:2078 (2001)), I declare:

1. I received a Bachelor of Science degree from Rensselaer Polytechnic Institute, a Ph.D. in Psychology with highest distinction from The Johns Hopkins University, and an M.D. from Yale University School of Medicine. I have been Director of the Developmental Biopsychiatry Research Program at McLean Hospital since 1988. For the

last decade, I have served as Associate Professor of Psychiatry at Harvard Medical School and Chief of the Developmental Psychopharmacology Laboratory at the Mailman Research Center. I am a member of the Editorial Board of the Journal of Child and Adolescent Psychopharmacology and have been a member of Harvard University's Brain Development Working Group. I have served on or chaired numerous review committees for the National Institutes of Health, published more than 150 articles, and have received numerous honors.

2. I have reviewed the papers, "*Dextran-methylprednisolone succinate as a prodrug of methylprednisolone: plasma and tissue disposition*", by Zhang and Mehvar, published in J. Pharm. Sci. 90:2078 (2001) and "*Kinetics of hydrolysis of dextran-methylprednisolone succinate, a macromolecular prodrug of methylprednisolone, in rat blood and liver lysosomes*", by Mehvar, Dann, and Hoganson, published in J. Control. Release 68:53 (2000) to answer the question of whether the dextran-methylprednisolone succinate conjugate is resistant to *in vivo* cleavage, such that *in vivo* less than 10% of the administered corticosteroid conjugate is cleaved prior to excretion. It is my opinion that the data provided by these two articles shows that the dextran-methylprednisolone succinate conjugate is not resistant to *in vivo* cleavage as required by the pending claims.

3. Dextran - methylprednisolone succinate (DMP) was designed to be a prodrug of methylprednisolone (MP). Hence, DMP is by itself inert, and it only becomes activated

after it has been cleaved to MP. As Zhang & Mehvar (2001) state on page 2084, *"Recent studies[29] in our laboratory demonstrated that DMP by itself lacks a significant immunosuppressive activity and should release MP to be effective."* A major focus of the Zhang & Mehvar (2001) paper was to document that systemic administration of DMP resulted in significant concentrations of cleaved MP in spleen and liver. The paper by Mehvar, Dann and Hoganon (2000) provided specific information on the hydrolysis of DMP in blood. DMP was converted to MP either directly or through cleavage to methylprenisolone succinate (MPS), which was rapidly converted to MP. DMP was hydrolyzed in blood with a rate constant of 0.028 h^{-1}, and a half-life of approximately 25 hours. Based on this information, 1% of the DMP would be converted in 30 minutes and 10% would be converted in 5 hours to MP and MPS. Zhang & Mehvar (2001) report that DMP has a very slow in vivo clearance after systemic administration, measured at 0.413 ml/min per kg in rats. Overall, in view of the very slow clearance of DMP from blood and tissues and the reported hydrolysis rate for DMP it is my opinion that at least 50% of the DMP conjugate would be cleaved prior to clearance.

4. All statements made herein of my own knowledge are true and all statements made on information and belief are believed to be true; and further these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United

States Code and that such willful false statements may jeopardize the validity of the application or any patents issued thereon.

5/29/06
Date

Martin H. Teicher, M.D., Ph.D.

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Kinetics of hydrolysis of dextran–methylprednisolone succinate, a macromolecular prodrug of methylprednisolone, in rat blood and liver lysosomes

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Abstract

A macromolecular prodrug of methylprednisolone (MP) was synthesized by conjugating MP with dextran with a M_w of 70 000 through a succinic acid linker. It has been shown previously that the dextran–MP conjugate (DMP) releases MP directly or indirectly through formation of methylprednisolone succinate (MPS) which is further hydrolyzed to MP. To investigate the suitability of DMP conjugate as a prodrug of MP for systemic administration, the kinetics of hydrolysis of the conjugate was studied in vitro in rat blood and liver lysosomes. In blood, the hydrolysis of MPS to MP was ~ten-fold faster than that in buffer. However, the hydrolysis rate constants of DMP conjugate to MP or MPS in blood were not different from those in buffer. Overall, the hydrolysis of DMP in the rat blood occurred with a half life of ~25 h. Hydrolysis of MPS to MP also occurred in the liver lysosomal fraction, but not in the control samples lacking lysosomes. However, the rate constants for the hydrolysis of DMP conjugate to MP and MPS in the lysosomal fraction were not significantly different from those in the control samples. These data suggest that the slow hydrolysis of DMP conjugate to MP or MPS in both rat blood and liver lysosomes occurs mostly, if not completely, via chemical hydrolysis. However, the conversion of MPS to MP is apparently enzymatic. The data may have significant implications for systemic administration of the prodrug.

Keywords: Methylprednisolone succinate; Methylprednisolone; Methylprednisolone prodrugs; Blood hydrolysis; Lysosomes

1. Introduction

Methylprednisolone (MP) is a glucocorticoid which is used in a variety of diseases, including prevention of rejection in organ transplantation. The use of steroids and other immunosuppressants in organ transplantation, however, has been associated with significant toxicity [1–3]. Therefore, it is desirable to devise strategies to target immunosuppressants to their site of actions, including the transplanted organ [4]. For example, in liver transplantation, it would be desirable to direct the immunosuppressant agent to the liver for local immunosuppression [5].

Dextrans are glucose polymers which have been tested for directing anticancer drugs to the tumor

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tissue through passive accumulation of the dextran–anticancer conjugate in the tumor [6,7]. Additionally, conjugates of dextrans with corticosteroids have been tested previously for the purpose of local delivery of steroids in the colon as anti-inflammatory agents [8,9]. We have previously demonstrated that dextran polymers with a molecular weight (M_w) of ~70 000 significantly accumulate in the liver of rats [10,11]. Therefore, we hypothesized that after its systemic administration, MP conjugated to dextran (DMP) would accumulate in the liver and gradually releases the active drug, resulting in sustained effects and fewer side effects in liver transplantation. Consequently, it is necessary to determine the kinetics of release of MP from DMP conjugate in both the systemic circulation (blood) and the liver. Because dextrans reportedly accumulate in the liver lysosomes [12], the present study was designed to investigate the hydrolysis of DMP conjugate in the rat liver lysosomes, in addition to rat blood.

2. Materials and methods

2.1. Materials

Dextran with an average M_w of 73 000 and 6α-methylprednisolone (MP) were obtained from Sigma Chemical (St. Louis, MO, USA). 6α-Methylprednisolone 21-hemisuccinate (MPS) was purchased from Steraloids (Wilton, NH, USA). For chromatography, HPLC grade acetonitrile (Mallinckrodt Chromar HPLC) was obtained from VWR Scientific (Minneapolis, MN, USA). All other reagents were analytical grade and obtained through commercial sources.

2.2. Synthesis of DMP

The macromolecular prodrug of MP was synthesized by modification of a previously reported method [8] as described before [13]. Briefly, 0.3 g of MPS was dissolved in 3 ml of dimethylsulfoxide (DMSO), and 360 mg 1,1′-carbonyldidmidazole was added and allowed to react with MPS at room temperature for 30 min. Next, 40 ml of a 5% (w/v) solution of dextran in DMSO and 3.5 ml of triethylamine were added, and the mixture was left at room temperature for 24 h. This procedure results in a conjugate of dextran and MP with succinic acid as a linker (Fig. 1). The DMP conjugate was then purified [8] and stored at −20°C as a powder. The purity and degree of substitution of DMP conjugate were then determined using a size-exclusion chromatographic method described before [13]. The MP and MPS impurities in the conjugate powder were less than 0.1% (w/w) and the degree of substitution of the powder was 8% MP (w/w).

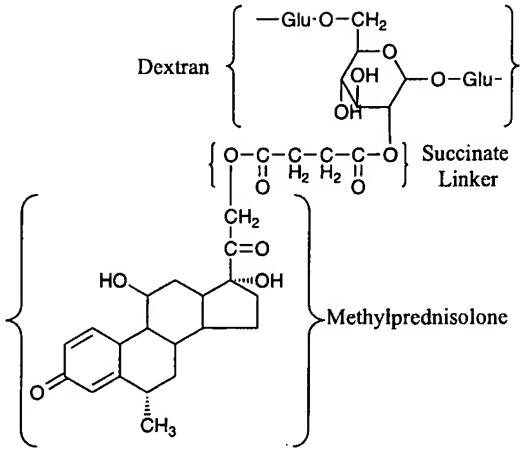

Fig. 1. Chemical structure of dextran–methylprednisolone hemisuccinate.

2.3. Blood hydrolysis

Blood was obtained from adult male Sprague–Dawley rats by cardiac puncture. Approximately 4 IU of heparin was added to each ml of blood to prevent coagulation. A preliminary study on the effect of time on the hydrolysis of DMP in blood indicated a slow hydrolysis of DMP conjugate in blood; after 3 h, ~5% of the prodrug was hydrolyzed. Therefore, further studies were designed to determine the initial rate of hydrolysis of DMP at different concentrations of the prodrug.

Immediately after the collection of blood, DMP conjugate (in isotonic phosphate buffer at pH 7.4) was added to produce blood concentrations of 5, 10, 25, 50, 75, and 100 μg/ml (MP equivalent) (n=3). After mixing blood with the DMP solution, a sample (1 ml) was taken for determination of baseline concentrations of the analytes. A second sample was

taken after the blood samples were incubated at 37°C in a shaking waterbath for 3 h. The blood samples were immediately centrifuged at 4°C, and the resultant plasma was used for the analysis of the concentrations of DMP conjugate or free MP and MPS. For control, similar samples were prepared in isotonic phosphate buffer, instead of blood, and subjected to procedures identical to those for the blood samples.

Separate experiments were conducted for determination of the hydrolysis kinetics of MPS conjugate in blood. However, for MPS, the concentrations used were 1, 2.5, 5, 7.5, and 10 μg/ml (MP equivalent) (n=3), and the incubation time was 1 h.

2.4. Liver lysosome hydrolysis

Lysosomes were isolated from adult male Sprague–Dawley rats (200–225 g) according to the method of Trouet [14] with minor modifications. Briefly, rats were injected intraperitoneally with a single 850-mg/kg dose of Triton WR-1339 four days before removal of the livers [15]. The livers were then homogenized in 0.25 M sucrose and subjected to differential centrifugation which resulted in collection of a lysosomal fraction. The potency of the lysosomal fraction was tested by the activity of acid phosphatase which is the marker for the lysosomes [14]. The specific activity of the enzyme was calculated, in both the liver homogenate and the lysosomal fraction, as the ratio of the enzyme activity per mg of protein. The protein contents of the preparations were determined by the Bio-Rad protein assay (Bio-Rad, Hercules, CA, USA). The activity of acid phosphatase was measured using a colorimetric assay [14]. In agreement with previous studies [14], the specific enzyme activity in the lysosomal fraction was >30-fold of that in the homogenate. The lysosomal fraction was stored at −80°C and used within one week of preparation.

For lysosomal hydrolysis studies (n=3), 1 ml of DMP conjugate or MPS solution in 0.2 M acetate buffer (pH 4.6) was added to 1 ml of the lysosomal fraction, and the samples were incubated at 37°C in a shaking waterbath for 12 h. This incubation time was based on a preliminary study indicating that the hydrolysis of DMP in the liver lysosomal fraction was measurable only with incubation times of 12 h. The final concentrations (MP equivalent) in the incubation media were 50, 100, and 200 μg/ml for DMP conjugate and 2.5, 5.0, and 10 μg/ml for MPS. Samples were taken at time zero (before the incubation) and at 12 h after the incubation and used for the analysis of DMP conjugate and free MPS and MP concentrations. For controls, similar samples were prepared by addition of drug solutions to 0.25 M sucrose (instead of lysosomal fraction), and subjected to the procedures identical to those for the lysosome samples.

2.5. Sample handling and storage

Plasma or lysosomal samples and their respective controls were divided into two portions, one for the analysis of free MP and MPS and the other for the analysis of DMP conjugate. The samples for the free MP and MPS were added to prechilled glass test tubes containing 100 μl of 10% (v/v) glacial acetic acid and stored at −20°C for the same day analysis. The samples for the analysis of DMP conjugate were added to prechilled siliconized microcentrifuge tubes and stored at −80°C for analysis within one week.

2.6. Analytical methods

The concentrations of MPS and MP in the samples were determined by a reversed-phase HPLC method described before [16].

A modified version of a size-exclusion chromatographic method reported before [13] was used for the quantitation of the concentrations of DMP in the samples. Briefly, after the precipitation of the sample (100 μl) proteins, with 20% (v/v) perchloric acid, DMP conjugate was analyzed using a gel chromatography column (Polysep-GFC; Phenomenex, Torrance, CA, USA) with a mobile phase of water:acetonitrile:glacial acetic acid (75:25:0.2) and a flow-rate of 1 ml/min. The samples were detected using a UV detector at a λ of 250 nm.

2.7. Data analysis

The hydrolysis of DMP conjugate to MP and MPS

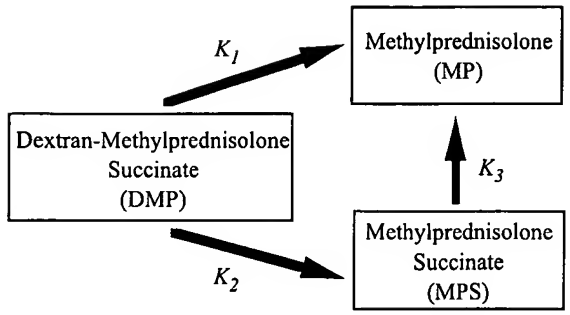

Fig. 2. The release scheme of MPS and MP from the prodrug along with relevant rate constants.

occurs according to Fig. 2 [8] and the following equations:

$$\frac{dMPS}{dt} = K_1 DMP + K_3 MPS \quad (1)$$

$$\frac{dMP}{dt} = K_2 DMP - K_3 MPS \quad (2)$$

where dMP/dt and $dMPS/dt$ are the rate of appearance of MP and MPS in the sample, respectively. Additionally, the following equation describes the hydrolysis of MPS:

$$\frac{dMP}{dt} = K_3 MPS \quad (3)$$

After the incubation of DMP conjugate or MPS, dMP/dt and/or $dMPS/dt$ were determined by dividing the generated concentration of MP or MPS by the incubation time. The rate constant for hydrolysis of MPS to MP (K_3) in blood or the lysosomal fraction (and their respective controls) was determined directly after the incubation of MPS by dividing dMP/dt by the initial MPS concentration in the sample. The rate constants for the hydrolysis of DMP conjugate to MP (K_1) or MPS (K_2) were then estimated using non-linear regression analysis of Eqs. (1) and (2).

The statistical differences between the estimated rate constants for blood and buffer or for the lysosomes and sucrose were determined using factorial ANOVA with post-hoc analysis of the means using the Scheffe F test at a significance level of 0.05. The data are presented as mean±S.D.

3. Results

The synthesized macromolecular prodrug had a degree of substitution of ~8% (w/w) MP with virtually no detectable impurities of MP or MPS.

The rates of appearance of MP (dMP/dt) in the rat blood and buffer samples incubated with MPS are shown in Fig. 3. As demonstrated in this figure, there was a linear relationship between dMP/dt and MPS concentration in blood samples, indicating a first-order release of MP from MPS in the rat blood. The rate of release of MP from MPS in the buffer, however, was very negligible and significantly less than that in blood (Fig. 3).

The rates of appearance of MPS and MP from DMP conjugate in the rat blood are presented in Fig. 4. Similar to the conversion of MPS to MP (Fig. 3), the appearance of MPS and MP after incubation of DMP (Fig. 4) is apparently governed by first-order processes (linear relationships between the rate of appearance and DMP concentration). For MPS, the rate appears to be slightly higher in the buffer than in blood (Fig. 4, top). However, for MP, the rates are almost superimposable in the buffer and blood (Fig. 4, bottom).

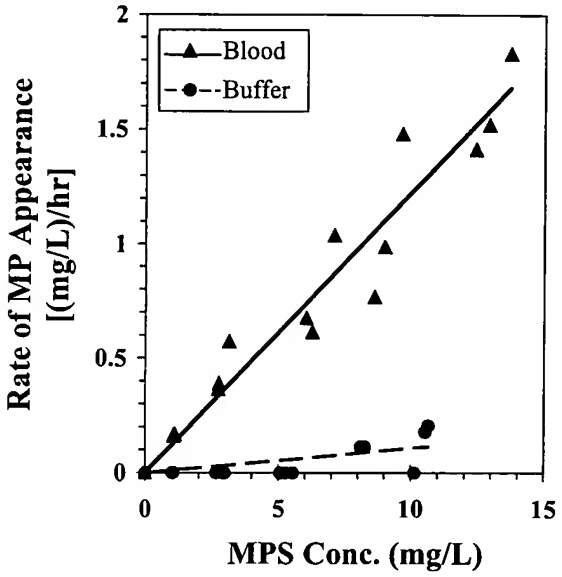

Fig. 3. The rate of appearance of methylprednisolone after the incubation of methylprednisolone succinate in rat blood or buffer.

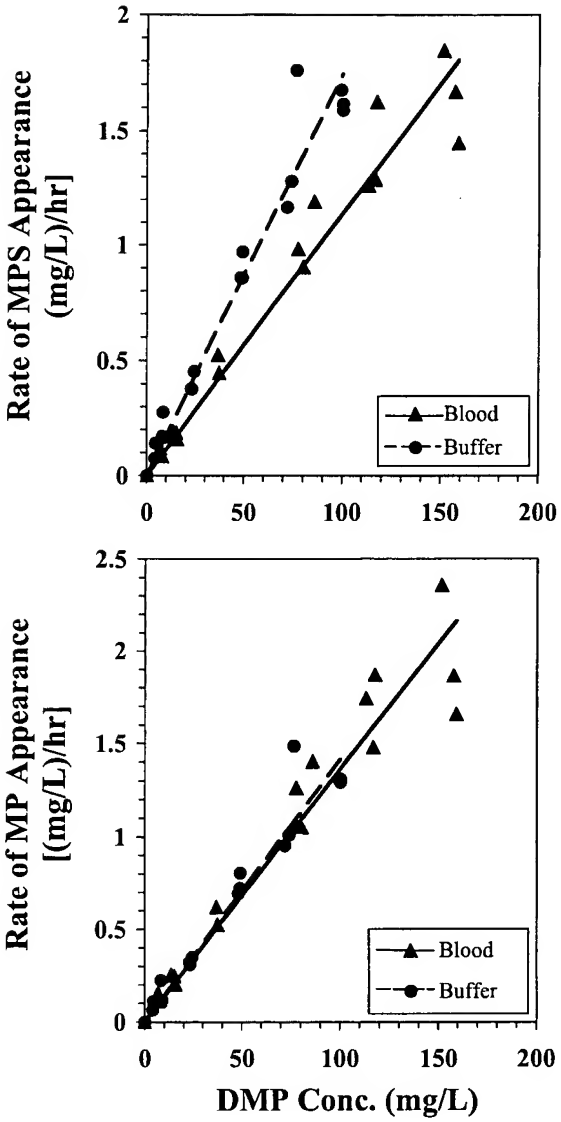

Fig. 4. The rate of appearance of methylprednisolone succinate (top) or methylprednisolone (bottom) after the incubation of the prodrug in rat blood or buffer.

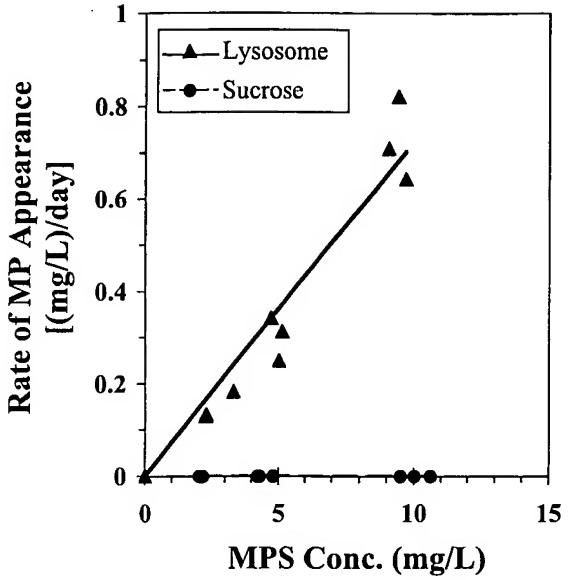

Fig. 5. The rate of appearance of methylprednisolone after the incubation of methylprednisolone succinate in rat liver lysosomes or sucrose.

Fig. 5 illustrates a linear relationship between the rate of MP appearance and MPS concentrations in the presence of liver lysosomes. However, no detectable concentrations of MP were observed when lysosomes were absent from the media (Fig. 5).

The rates of appearances of MPS and MP after the incubation of DMP conjugate in liver lysosomes are presented in Fig. 6. Additionally, the rates after the incubation with sucrose (controls) are demonstrated in this figure. While the rates of MPS appearance in the presence and absence of lysosomes were superimposable (Fig. 6, top), lysosomal incubations produced slightly higher rates of appearance for MP (Fig. 6, bottom).

The estimated rate constants in rat blood and buffer are presented in Table 1. In blood, the rate constant for hydrolysis of MPS to MP (K_3) was approximately ten-fold higher than that for the hydrolysis of DMP to MP (K_1) or to MPS (K_2) (Table 1). Although there was a significant difference between blood and buffer in K_3, blood and buffer values of K_1 and K_2 were similar (Table 1).

The rate constant values in the presence of the lysosomal fraction and sucrose alone are listed in Table 2. In general, the lysosomal rate constants (Table 2) were several fold smaller than their corresponding values in blood (Table 1). However, the qualitative differences among rate constants and between the lysosomes and controls were similar to those in blood; the rate constant for the hydrolysis of MPS to MP (K_3) was >20-fold higher than the

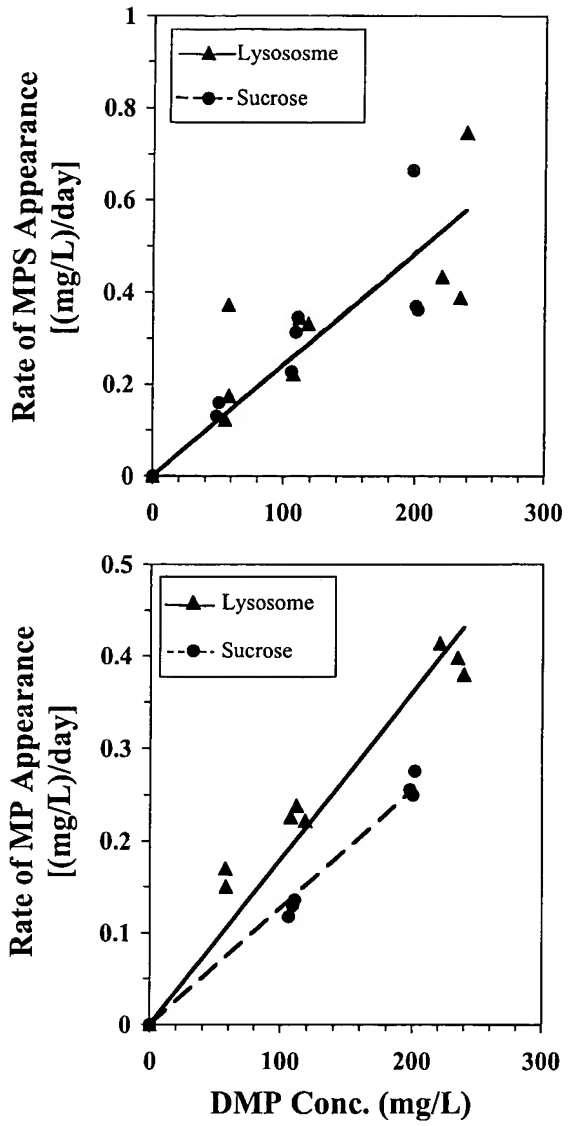

Fig. 6. The rate of appearance of methylprednisolone succinate (top) or methylprednisolone (bottom) after the incubation of the prodrug in rat liver lysosomes or sucrose.

constants for the hydrolysis of DMP (K_1 or K_2) (Table 2). Additionally, there were no significant differences between the lysosome and sucrose samples in their K_1 and K_2 values.

4. Discussion

4.1. Blood hydrolysis

The synthesized DMP conjugate (Fig. 1) can either release MP directly by cleavage of the ester bond adjacent to MP or indirectly through MPS, by cleavage of the ester bond adjacent to the dextran molecule (Figs. 1 and 2). The released MPS is then hydrolyzed to form MP (Fig. 2). The blood data (Table 1) indicate that the hydrolysis of MPS to MP is much (~ten-fold) faster in blood than in buffer, suggesting enzymatic hydrolysis of MPS by blood esterases. However, the much slower hydrolysis of either ester bonds of DMP conjugate (K_1 or K_2), compared with MPS (K_3), suggests that blood esterases cannot act on DMP conjugate. Similar observations were made when dextran–steroid conjugates or MPS was incubated with luminal contents of the upper gastrointestinal tract of rats [9]; while MPS was rapidly hydrolyzed by upper gastrointestinal tract esterases, dextran–steroid conjugates remained intact. However, in the lower part of gastrointestinal tract, where the M_w of dextran polymer is reduced by dextranases, faster hydrolysis of dextran–steroids was observed [9]. Therefore, the slow hydrolysis of DMP conjugate in blood (Table 1) appears to be due to steric hindrance of the dextran macromolecule.

The slightly higher rate of appearance of MPS in buffer, compared with blood (Fig. 4, top), may be explained by the fact that the rate constant of hydrolysis of MPS to MP (K_3) was significantly lower in buffer (Table 1). Because the rate of

Table 1
Rate constants of hydrolysis of DMP conjugate to MP (K_1) and MPS (K_2) and that of MPS to MP (K_3) in rat blood

Constant (h^{-1})	K_1	K_2	K_3
Blood	0.0128±0.0025[a]	0.0147±0.0023[b]	0.133±0.025[a,b,c]
Buffer	0.0154±0.0041	0.0202±0.0052	0.0156±0.0025[c]

[a,b,c] Means with a similar letter are significantly different from each other.

Table 2
Rate constants of hydrolysis of DMP conjugate to MP (K_1) and MPS (K_2) and that of MPS to MP (K_3) in rat liver lysosomes

Constant (day^{-1})	K_1	K_2	K_3
Lysosomes	0.00204 ± 0.00044^a	0.00307 ± 0.00152^b	$0.0649 \pm 0.0122^{a,b,c}$
Sucrose	0.00124 ± 0.00009	0.00253 ± 0.00068	0^c

[a,b,c] Means with similar letters are significantly different from each other.

appearance of MPS is a net function of both formation from DMP conjugate and further hydrolysis to MP [Eq. (2)], in the presence of similar formation rates, a lower hydrolysis rate in buffer would result in a higher appearance of MPS in this media (Fig. 4, top). The rate of formation of MP, however, was the same for blood and buffer after the incubation of DMP conjugate (Fig. 4, bottom).

In their studies of dextran–steroid conjugates for colon-specific delivery, McLeod et al. [8] investigated DMP hydrolysis rate constants in a pH 6.8 buffer at 37°C. Our estimated rate constants in pH 7.4 (Table 1) are two–four-fold higher than those reported by these authors at pH 6.8. This is due, perhaps, to the fact that the hydrolysis rate constant of dextran–steroid conjugates substantially increases with an increase in pH [8].

One of the problems associated with ester prodrugs is rapid in vivo hydrolysis of the prodrug in blood before the achievement of appropriate concentrations of the prodrug at target tissue(s). Our in vitro blood hydrolysis data (Table 1), however, suggest that the hydrolysis of DMP conjugate in blood is slow with a rate constant of 0.028 h^{-1}($K_1 + K_2$) and a corresponding half life of ~25 h. The relatively slow hydrolysis of DMP conjugate in blood should allow distribution of DMP to its target sites (including the liver) after its systemic administration.

Another potential problem dealing with the pharmacokinetics of ester prodrugs is the ex vivo ester hydrolysis after blood is collected from humans or animals and during the sample preparation and storage. This is especially important if the prodrug concentration in the sample is much larger than that of the drug. In these cases, ex vivo hydrolysis of a small fraction of prodrug can substantially affect the concentrations and estimated pharmacokinetics of the drug. For example, our estimated rate constant of 0.028 h^{-1} for the hydrolysis of DMP conjugate in blood means that within 0.5 h after the blood collection, approximately 1% of DMP could be hydrolyzed. Assuming a DMP concentration of 100 μg/ml, this hydrolysis means ex vivo production of 1 μg/ml MP and/or MPS. Considering the expected low plasma concentrations of MP and MPS in vivo, this ex vivo hydrolysis can adversely affect the calculation of pharmacokinetic parameters. Therefore, further experiments were conducted to devise methods to prevent ex vivo hydrolysis of DMP during sample collection and hydrolysis. These studies (data not shown) indicated that if blood samples are immediately centrifuged in prechilled glass test tubes at 4°C and plasma is analyzed immediately, no hydrolysis products (MP or MPS) could be detected. Additionally, it was shown that when plasma samples (0.5 ml) are added to prechilled glass tubes containing 100 μl of 10% (v/v) glacial acetic acid and stored at −80°C, no hydrolysis product could be detected up to 3 weeks after the storage. Therefore, future studies of the kinetics of DMP conjugate and released MP and MPS will follow this sample collection and storage protocol to avoid pitfalls in calculating pharmacokinetic parameters.

4.2. Lysosome hydrolysis

The hydrolysis of MPS in the lysosomal fraction and lack of hydrolysis in the control samples (Fig. 5) are consistent with the reported [17] presence of esterases in the lysosomal fraction. However, the MPS to MP hydrolysis rate constant in the lysosomal fraction (0.0649 ± 0.0122 day^{-1}) is ~50-fold smaller than that in blood (0.133 ± 0.025 h^{-1}), suggesting that the levels of esterases in the lysosomal fraction are lower than those in blood.

Similar to blood, the rate constants of hydrolysis of DMP conjugate to MP (K_1) and MPS (K_2) in the lysosomal fractions were not significantly different

than those in the control samples (Table 2), suggesting that the very slow hydrolysis of DMP conjugate in lysosomes is through chemical, rather than enzymatic, hydrolysis. Overall, the rate constant of hydrolysis of DMP in the lysosomes (K_1+K_2; 0.0051 day^{-1}) was more than 100-fold smaller than that in blood (0.028 h^{-1}). This difference is mostly, if not completely, due to a lower pH value used in the lysosomal studies (4.6), compared with the pH value for the blood hydrolysis experiments (7.4); previous studies [8] have shown that the hydrolysis of DMP conjugate substantially decreases as the pH of the media is reduced. Nevertheless, the in vitro hydrolysis of DMP conjugate in the lysosomal fraction appears to be very slow with a half life of more than 100 days.

In addition to esterases and other enzymes, the liver lysosomes contain dextranases [17] which are expected to reduce the M_w of dextrans and their derivatives. A reduction in the M_w of the dextran carrier in the lysosomes is then expected to make the ester bond more susceptible to the action of esterases in this compartment. In agreement with this hypothesis, a reduction in the M_w of dextrans by dextranases in the colon has been held responsible for the hydrolysis of the conjugates of dextrans with steroids [9] or with nonsteroidal anti-inflammatory agents [18] by colon esterases. However, it should be noted that, in contrast to the lysosomal dextranases, the colon dextranases are of bacterial origin. Therefore the cleavage of dextrans in the colon and liver may not be similar.

In our liver lysosomal studies, we did not detect any substantial differences between the lysosomal fraction and the controls (sucrose) for hydrolysis of DMP conjugate (Table 2). The lack of a significant enzymatic hydrolysis in the lysosomal fraction may be due to a lack of a substantial reduction in the M_w of DMP conjugate during our hydrolysis experiments. Indeed, our size-exclusion chromatographic method could not detect any noticeable change in the retention times of the DMP samples before and after lysosomal incubation, suggesting no significant change in the M_w of DMP conjugate during the incubation period. This is in agreement with the results reported by Chiu et al. [19], failing to demonstrate degradation of dextrans in liver lysosomal fractions in vitro. However, these in vitro results should be extrapolated to in vivo situations with caution. This is because the lysosomal fraction obtained by the Triton method contains a substantial amount of Triton and some proteins [14] which may affect the metabolism of other drugs. Additionally, in vivo studies in rats [11] and mice [20] have demonstrated a significant reduction in the molecular weight of dextran residing in the liver. Nevertheless, future studies, investigating the in vivo release and effects of MP after the administration of DMP conjugate are needed to determine whether DMP conjugate releases MP in the liver in a sufficient and slow manner to produce a sustained immunosuppressive effect in this organ.

5. Conclusions

In conclusion, the kinetics of hydrolysis of DMP conjugate to MP and MPS and that of MPS to MP were determined in rat blood and liver lysosomal fraction. In both media, the conversion of MPS to MP occurred through enzymatic hydrolysis. However, the conversion of DMP conjugate to MPS and MP was very slow and appeared to be driven by chemical hydrolysis. The slow hydrolysis of DMP conjugate in blood is expected to allow distribution of DMP to tissue(s) of interest after its systemic administration. However, it remains to be seen whether the release of MP in the liver lysosomal fraction after its systemic administration is sufficient for production of local immunosuppression in the liver.

Acknowledgements

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